

10048186.061902

JC07 Rec'd PCT/PTO 25 JAN 2002

SUBSTITUTE FORM PTO-1390

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER  
06497-013002

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)

10/048186

INTERNATIONAL APPLICATION NO.  
PCT/US00/20519INTERNATIONAL FILING DATE  
27 July 2000PRIORITY DATE CLAIMED  
27 July 1999TITLE OF INVENTION  
ENGINEERING OF METABOLIC CONTROLAPPLICANT(S) FOR DO/EO/US  
James C. Liao

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☒ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11 to 16 below concern other documents or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
  - ☒ International Search Report
  - ☐
  - ☐
  - ☐
  - ☐

## CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EL 48578174805

I hereby certify under 37 CFR §1.10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, DC 20231

1-25-02  
Date of Deposit

Signature

Typed Name of  
Person Signing

|  |              |   |   |  |    |
|--|--------------|---|---|--|----|
| U.S. APPLICATION NO. (IF KNOWN)<br><b>10/048186</b>  |              | INTERNATIONAL APPLICATION NO.<br>PCT/US00/20519 |   | ATTORNEY'S DOCKET NUMBER<br>06497-013002 |    |
| 17. <input checked="" type="checkbox"/> The following fees are submitted:<br><b>Basic National Fee ( 37 CFR 1.492(a)(1)-(5) ):</b><br><br>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... <b>\$1040</b><br><br>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890</b><br><br>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740</b><br><br>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710</b><br><br>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100</b><br><br><div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div> |              |   |   | <b>CALCULATIONS PTO USE ONLY</b>         |    |
|  |              |   |   |  |    |
|  |              |   |   |  |    |
|  |              |   |   |  |    |
|  |              |   |   |  |    |
| Surcharge of <b>\$130</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).  |              |   |   | \$0.00                                   |    |
| Claims   | Number Filed | Number Extra                                    | Rate  |  |    |
| Total Claims   | 35 - 20 =    | 15  | x <b>\$18</b>   | \$270.00                                 |    |
| Independent Claims   | 6 - 3 =      | 3   | x <b>\$84</b>   | \$252.00                                 |    |
| MULTIPLE DEPENDENT CLAIMS(S) (if applicable)   |              |   | + <b>\$280</b>  | \$0.00                                   |    |
| <b>TOTAL OF ABOVE CALCULATIONS =</b>   |              |   |   | \$1,232.00                               |    |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.   |              |   |   | \$0.00                                   |    |
| <b>SUBTOTAL =</b>  |              |   |   | \$1,232.00                               |    |
| Processing fee of <b>\$130</b> for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))  |              |   |   | \$0.00                                   |    |
| <b>TOTAL NATIONAL FEE =</b>  |              |   |   | \$0.00                                   |    |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +  |              |   |   | \$0.00                                   |    |
| <b>TOTAL FEES ENCLOSED =</b>   |              |   |   | \$1,232.00                               |    |
|  |              |   |   | <b>Amount to be refunded:</b>            | \$ |
|  |              |   |   | <b>Charged:</b>                          | \$ |
| a. <input checked="" type="checkbox"/> A check in the amount of \$1,232.00 to cover the above fees is enclosed.<br>b. <input type="checkbox"/> Please charge my Deposit Account No. 06-1050 in the amount of \$0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.<br>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.  |              |   |   |  |    |
| <b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.</b>  |              |   |   |  |    |
| SEND ALL CORRESPONDENCE TO:  |              |   |   |  |    |
| Y. Rocky Tsao<br>FISH & RICHARDSON P.C.<br>225 Franklin Street<br>Boston, Massachusetts 02110-2804<br>(617) 542-5070 phone<br>(617) 542-8906 facsimile   |              |   | <div style="text-align: center;"> <br/>           SIGNATURE:         </div> <div style="text-align: center;">           NAME: Y. Rocky Tsao         </div> <div style="text-align: center;">           REGISTRATION NUMBER: 34,053         </div> |  |    |

## ENGINEERING OF METABOLIC CONTROL

### *Background of the Invention*

The use of recombinant DNA technology has allowed the engineering of host cells to produce desired compounds, such as polypeptides and secondary metabolites. The large scale production of polypeptides in engineered cells allows for the production of proteins with pharmaceutical uses and enzymes with industrial uses. Secondary metabolites are products derived from nature that have long been known for their biological and medicinal importance. Because of the structural complexity inherent in such molecules, traditional chemical synthesis often requires extensive effort and the use of expensive precursors and cofactors to prepare the compound. In recent years, the expression of heterologous proteins in cells has facilitated the engineering of heterologous biosynthetic pathways in microorganisms to produce metabolites from inexpensive starting materials. In this manner, a variety of compounds have been produced, including polyketides,  $\beta$ -lactam antibiotics, monoterpenes, steroids, and aromatics.

### *Summary of the Invention*

The invention is based, in part, on the discovery that production of heterologous polypeptides and metabolites can be enhanced by the regulated expression of the polypeptide (e.g., a biosynthetic enzyme) using a promoter which is regulated by the concentrations of a second metabolite, e.g. acetyl phosphate. The term "heterologous" refers to a polypeptide or metabolite which is introduced by artifice. A heterologous polypeptide or metabolite can be identical to endogenous entity that is naturally present. The term "metabolite" refers to a organic compound which is the product of one or more biochemical reactions. A metabolite may itself be a precursor for other reactions. A secondary metabolite is a metabolite derived from another.

Accordingly, in one aspect, the invention features a bacterial host cell containing a nucleic acid sequence comprising a promoter and a nucleic acid sequence encoding a heterologous polypeptide. Examples of bacterial host cells include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *Agrobacterium tumefaciens*, *Thermus thermophilus*, and *Rhizobium leguminosarum* cells. The nucleic acid sequence is operably linked to the promoter which is controlled by a response regulator protein. In other words, the nucleic acid

sequence is linked to the promoter sequence in a manner which allows for expression of the nucleotide sequence in vitro and in vivo. "Promoter" refers to any DNA fragment which directs transcription of genetic material. The promoter is controlled by a response regulator protein, for example, ntrC, phoB, phoP, ompR, cheY, creB, or torR, of *E. coli* or its homologs from other bacterial species. Further, the response regulator protein can be another member of the cluster orthologous group (COG) COG0745 as defined by <http://www.ncbi.nlm.nih.gov/COG/> (Tatusov *et al. Nucleic Acids Res.* (2000); 28:33-36). In one implementation, the promoter is bound by *E. coli* ntrC. The term "ntrC" refers to both the *E. coli* ntrC protein (SWISSPROT : P06713, <http://www.expasy.ch/>) and its homologs in other bacteria as appropriate. As used herein, "bound" refers to a physical association with a equilibrium binding constant ( $K_D$ ) of less than 100 nM, preferably less than 1 nM. An example of the promoter is the *E. coli glnAp<sub>2</sub>* promoter, e.g. a region between positions about 93 and about 323 in the published DNA sequence, GenBank accession no. M10421 (Reitzer & Magasanik (1985) *Proc Nat Acad Sci USA* 82:1979-1983). This region includes untranslated sequences from the *glnA* gene. Further, a translational fusion can be constructed between coding sequences for *glnA* and coding sequences for the heterologous polypeptide.

The host cell is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation. For example, the host cell can genetically modified by deletion or mutation of a gene encoding a histidine protein kinase, e.g., a member of COG0642 as defined by (<http://www.ncbi.nlm.nih.gov/COG/>; Tatusov *et al. supra.*), e.g., *glnL*, *phoR*, *phoQ*, *creC*, or *envZ*. In another example, the histidine protein kinase has specificity for the response regulator protein which controls the promoter. The histidine protein kinase can be encoded by *glnL*, e.g., *E. coli glnL* (SWISSPROT P06712; <http://www.expasy.ch/>).

Whereas the host cell is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation, for heterologous polypeptide or metabolite expression, the host cell can be propagated in any desired condition, e.g., in nitrogen starvation conditions, nitrogen poor conditions, or nitrogen rich conditions.

The heterologous polypeptide encoded by the nucleic acid sequence can be a biosynthetic enzyme required for production of a metabolite. It can be a mammalian protein, e.g., a secreted growth factor, a monoclonal antibody, or an extracellular matrix component.

In yet another example, the heterologous polypeptide can be a desired antigen for use in a vaccine, e.g., a surface protein from a viral, bacterial, fungal, or protist pathogen.

Another aspect of the invention features a kit containing a nucleic acid sequence which includes a promoter controlled by a response regulator protein. The kit further  
 5 optionally contains a bacterial host cell which is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation. The kit can also provide instructions for their use. The nucleic acid sequence can contain a restriction enzyme polylinker located 3' of the promoter such that a sequence inserted into the polylinker is operably linked to the promoter which is controlled by a response regulator protein. In one  
 10 implementation of the kit, the promoter is the *E. coli glnAp<sub>2</sub>* promoter and the bacterial host cell is an *E. coli* cell containing a mutation or deletion of the *glnL* gene.

Another aspect of the invention features a host cell containing a first expression cassette. The first expression cassette includes a promoter, such as any of those described above, and a nucleic acid sequence encoding an enzyme required for biosynthesis of a  
 15 heterologous metabolite. As used herein, "enzyme" refers to a polypeptide having ability to catalyze a chemical reaction or multiple reactions. The nucleic acid sequence is operably linked to the promoter which is regulated by acetyl phosphate in the absence of nitrogen starvation. The host cell also contains additional nucleic acid sequences for expressing other enzymes required for biosynthesis of the metabolite. Such additional sequences may be  
 20 endogenous sequences expressing endogenous enzymes, or introduced sequences expressing heterologous enzymes.

In one example, the heterologous metabolite is an isoprenoid, a polyhydroxyalkanoate, a polyketide, a  $\beta$ -lactam antibiotic, an aromatic, or a precursor, e.g., an upstream metabolite, or a derivative, e.g., a downstream metabolite, thereof. For instance,  
 25 the isoprenoid can be a carotenoid, a sterol, a taxol, a diterpene, a gibberellin, and a quinone. Specific examples of isoprenoids include isopentenyl diphosphate, dimethylallyl diphosphate, geranyl diphosphate, farnesyl diphosphate, geranylgeranyl diphosphate, and phytoene. Specific examples of carotenoids include  $\beta$ -carotene,  $\zeta$ -carotene, astaxanthin, zeaxanthin, zeaxanthin- $\beta$ -glucoside, phytofluene, neurosporene, lutein, and torulene. When the desired  
 30 heterologous metabolite is an isoprenoid, the heterologous enzyme can be isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, or 1-deoxyxylulose 5-phosphate synthase. When the desired heterologous metabolite is an polyhydroxyalkanoate,

the heterologous enzyme can be 3-ketoacyl reductase, or poly-3-hydroxyalkanoate polymerase.

The host cell can be a bacterial cell, e.g., an *E. coli* cell. The host cell is optionally genetically modified by deletion or mutation of a gene, e.g., a gene encoding a histidine protein kinase, as described above. In one specific example, the host cell further contains a second expression cassette containing a nucleic acid sequence encoding phosphoenolpyruvate synthase operably linked to a promoter regulated by acetyl phosphate concentration, e.g., *glnAp<sub>2</sub>*.

Another aspect of invention features a method of producing heterologous isoprenoids in a host cell. The method includes overexpressing phosphoenolpyruvate synthase and expressing biosynthetic enzymes required for synthesis of the heterologous isoprenoid. In one implementation, a gene in the host cell encoding a pyruvate kinase or a phosphoenolpyruvate carboxylase is genetically deleted or enfeebled. In another implementation, a gene encoding phosphoenolpyruvate carboxykinase is overexpressed in the host cell. Still another aspect of the invention features a method of producing a lycopene in a host cell. The method includes expressing the following heterologous enzymes: 1-deoxy-D-xylulose 5-phosphate synthase, a geranylgeranyl diphosphate synthase, a phytoene synthase, and a phytoene saturase. In one implementation of this method, an isopentenyl diphosphate isomerase is overexpressed, e.g., using the *glnAp<sub>2</sub>* promoter. In another implementation, a phosphoenolpyruvate synthase is overexpressed, e.g., using the *glnAp<sub>2</sub>* promoter.

Another aspect of the invention features a nucleic acid sequence containing a promoter and a sequence encoding a biosynthetic enzyme required for the production of a first metabolite. The promoter is operably linked to the sequence, and is regulated by a second metabolite whose concentration is indicative of availability of a precursor for the biosynthesis of the first metabolite. In one example, the second metabolite is a waste product produced from a precursor for the biosynthesis of the first metabolite.

In one implementation, the first metabolite is a polyhydroxyalkanoate, e.g., polyhydroxybutyrate and the nucleic acid sequence encodes a biosynthetic enzyme, e.g., a 3-ketoacyl coenzyme A (coA) reductases, or a poly-3-hydroxyoctanoyl-CoA polymerase. In another case, the first metabolite is a polyketide, a  $\beta$ -lactam antibiotic, or an aromatic. In a yet another case, the first metabolite is an isoprenoid, e.g., an isoprenoid mentioned herein. The nucleic acid sequence can encode a biosynthetic enzyme required for isoprenoid

production, e.g., isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, 1-deoxyxylulose 5-phosphate synthase, phosphoenolpyruvate synthase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, or lycopene cyclase. One precursor of isoprenoids can be pyruvate. Pyruvate concentrations  
5 are related to acetate and acetyl-phosphate concentrations. Accordingly, in this instance, the second metabolite is acetyl phosphate. The promoter responding to acetyl phosphate can be controlled by a response regulator protein, e.g., a response regulator protein mentioned above. Such a promoter may only respond to acetyl phosphate in a specific host cell. In a particular example, the promoter responding to acetyl phosphate concentration is bound by *E.*  
10 *coli* ntrC, e.g., *E. coli* glnAp<sub>2</sub> promoter.

The promoter can be regulated by cAMP. The promoter can be a bacterial promoter which binds CAP (catabolite activator protein). In mammals, the promoter can be a promoter containing a cAMP response element (CRE), which binds to the proteins CREB, CREM, or ATF-1. In yeast cells, the promoter can be a promoter regulated by cAMP, or a promoter  
15 bound by proteins Gis1, Msn2, or Msn4. Another possible regulatory signal for the promoter can be fructose 1-phosphate, or fructose 6-phosphate. The *E. coli* FruR protein regulates such promoters.

The nucleic acid sequence can be contained on a plasmid. It can also contain a bacterial origin of replication and a selectable marker. The sequence can further contain a  
20 yeast or other eukaryotic origin of replication and appropriate selectable markers, and can be integrated into the genome.

The optimization of biosynthesis of heterologous compounds in host cells is reliant on sensing parameters of cell physiology and on utilizing these parameters to regulate the biosynthesis. One standard techniques in the art is to grow cells and for the user to  
25 exogenously add an agent, e.g., an inducer, to turn on genes required for biosynthesis of the desired product. It has been widely observed that high-level induction of a recombinant protein or pathway leads to growth retardation and reduced metabolic activity. (Kurland and Dong (1996) *Mol Microbiol* 21:1-4). The practice of exogenously supplying an inducer is empirical and does not monitor the availability of resources in the cell for biosynthesis. In  
30 contrast, natural pathways rely on feedback mechanisms to control such processes. The combination of certain promoters with specific genetically defined host cells and heterologous polypeptides in this invention unexpectedly results in a highly refined and

versatile control circuit that regulates flux to heterologous polypeptide or metabolite synthesis in response to the metabolic state of the cell. Indeed, the dynamically controlled recombinant pathway provides for enhanced production, minimized growth retardation, and reduced toxic by-product formation. The regulation of gene expression in response to physiological state will also benefit other applications, such as gene therapy.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

### *Detailed Description*

The invention provides methods of engineering metabolic control, e.g., methods of utilizing promoters in specific host cells in order to optimize protein expression for either protein production or metabolite synthesis.

A central component of the invention is an expression cassette comprising a promoter and nucleic acid sequence encoding a heterologous polypeptide whose expression is desired. The expression cassette is constructed using standard methods in the art such that the coding nucleic acid sequence is operably linked, e.g., regulated by, the promoter. The promoter is chosen such that the promoter is regulated by a parameter of cell physiology or cell metabolic state. A variety of promoters can be used. In some applications the expression cassette is contained within a plasmid, such as bacterial plasmid with a bacterial origin of replication and a selectable marker. The expression cassette can be integrated into the genome of cells using standard techniques in the art.

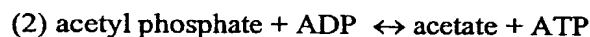
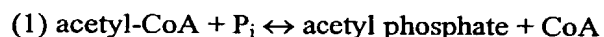
If the expression cassette is to be used for engineering regulated production of a heterologous polypeptide during late logarithmic growth or during stationary phase, then the promoter can be chosen accordingly. For example, a promoter can be chosen that responds to small molecule signal, e.g., a second messenger, whose levels accumulate during late logarithmic growth or during stationary phase. The second messenger can be a molecule that accumulates as a precursor, an intermediate, or a waste product of a biochemical pathway. In bacteria, the small molecule signal can be a glycolysis intermediate, e.g., fructose 1-phosphate or fructose 6-phosphate or a glycolysis waste product, e.g., acetate or acetyl



phosphate. In eukaryotic cells, cAMP concentrations are a well known signal of nutrient state.

The promoter in the expression cassette can be chosen based on the results of a large scale expression analysis experiment, e.g., a gene chip experiment. Genes which are induced  
 5 by acetyl phosphate can be identified by hybridizing to a microarray labeled cDNA prepared from cells in grown in acetate and comparing the signal to a reference signal, e.g., to the signal of obtained with cDNA prepared from cells in early logarithmic growth. This experiment can be performed on both prokaryotic and eukaryotic cells, e.g., bacterial, yeast, plant and mammalian cells. For an example of such an experiment in a prokaryote, see  
 10 Talaat et al. (2000) *Nat Biotechnol* 18:679-82 and Oh & Liao (2000) *Biotechnol Prog.* 16:278-86. Once a gene is identified which is expressed under the desired condition, its promoter can utilized in the expression cassette. Alternatively, the experiment can be performed by the exogenous addition of a desired molecule (e.g., a precursor in a metabolic pathway) or by manipulation of experimental conditions (e.g., growth to late logarithmic  
 15 phase or growth while a biosynthetic enzyme is overproduced). Promoters can be identified based on the genes induced.

In one instance, an expression cassette is used for engineering regulated production of a metabolite in a bacterial cell. The promoter can be selected which is regulated by a second metabolite whose concentration is indicative of the availability of a precursor for the  
 20 biosynthesis of the first metabolite. For example, if the first metabolite is an isoprenoid which is synthesized from the precursors, pyruvate and glyceraldehyde 3-phosphate, then the second metabolite can be acetyl phosphate. In a rich environment, cells produce an excess amount of acetyl-CoA, a product of pyruvate. The excess acetyl-CoA is used to produce ATP and acetate, which is secreted as a waste product. Acetate concentration increases with  
 25 cell density. Acetate, acetyl-CoA, and acetyl-phosphate concentrations are interrelated by to the following biochemical reactions:



Thus, high acetyl phosphate concentration is indicative of excess acetyl-CoA and  
 30 excess pyruvate. A host cell which is genetically modified by deletion or mutation of *glnL*, for example, causes *ntnC* function to become acetyl phosphate dependent (Feng *et al.* (1992) *J Bacteriol* 174:6061-6070). In this fashion, a promoter regulated by *ntnC*, e.g., the *glnAp2*

promoter, can be used to control gene expression in response to acetyl phosphate. The *glnAp2* promoter can be obtained using standard techniques in the art. For example, primers can be designed and synthesized that anneal to the *glnAp2* promoter. The polymerase chain reaction (PCR) can be used to amplify a nucleic acid fragment containing the *glnAp2* promoter. This fragment can now be used for further constructions. Likewise, an *E. coli* strain containing deletion of histidine protein kinase gene, e.g., *glnL* can be easily prepared. See Link *et al.* (1997) *J Bacteriol.* 179(20):6228-6237 for a detailed description of one possible method. The sequences encoding a desired heterologous polypeptide can be cloned downstream of the *glnAp2* promoter so that it is operably linked to the promoter. A host cell with an inactivated *glnL* gene can then be transformed with the sequences. The transformed strain can be grown, and polypeptide production monitored during the course of growth. Robust protein expression can be observed at high cell densities, as in Farmer and Liao (2000) *Nat. Biotechnol* 18:533-537, the contents of which are hereby incorporated by reference.

15           A mammalian cell can be used as a host cell for polypeptide or metabolite production. A promoter can be selected, e.g., a promoter that responds to cAMP. Such a promoter can contain a cAMP response element (CRE), which binds to the proteins CREB, CREM, or ATF-1. Using standard techniques in the art, a desired coding sequence can be placed under control of the promoter and transformed into the mammalian cell. In some instances, the construction can be inserted into a virus, e.g., an inactivated virus. Such implementations allow for the regulated production of a protein or a metabolite produced by a heterologous biosynthetic enzyme in a gene therapy scenario. Plant cells can also be used as host cells. Again, an appropriate promoter can be chosen, e.g., a promoter than responds to a plant hormone, metabolite, or a precursor for the production of a desired metabolite. A promoter can be identified by a microarray experiment. After fusion of a desired promoter to a desired coding sequence in an appropriate vector, the construction can be electroporated into *Agrobacterium tumefaciens* and then used to transform plant cells using standard methods in the art. In still another example, yeast cells can be manipulated to express heterologous polypeptides or metabolites under metabolic control. For example, a *Saccharomyces cerevisiae* promoter can be a promoter regulated by cAMP, e.g., a promoter bound by proteins Gis1, Msn2, or Msn4. The regulation of all yeast genes in response to a variety of metabolic conditions is increasingly well studied. For example, DeRisi *et al.* (1997) *Science*

278:680-686 describe experiments following the transcriptional profile of nearly the entire *Saccharomyces cerevisiae* gene set under various metabolic conditions. Promoters regulated by a desired metabolite can be selected based on such data. The generation of yeast plasmids and the transformation of yeast are well known in the art.

5 A variety of metabolic pathways can be reconstructed using the expression techniques described above. For example, a pathway to produce lycopene can be introduced in *E. coli* by constructing expression vectors for the following genes: *dxs* (coding for 1-deoxy-D-xylulose 5-phosphate synthase) from *E. coli*, *gps* (coding for geranylgeranyl diphosphate (GGPP) synthase) from *Archaeoglobus fulgidus*, and *crtBI* (coding for phytoene synthase and  
10 desaturase, respectively) from *Erwinia uredovora*. These genes can reside on a single or multiple plasmids, or can be integrated into the *E. coli* chromosome. In addition, phosphoenolpyruvate synthase can be overexpressed using any method, e.g., by fusion to the *glnAp2* promoter. Isopentyl diphosphate isomerase can be overexpressed using any method, e.g., by fusion to the *glnAp2* promoter.

15 In another example, a pathway to produce polyhydroxyalkanoates (PHA), e.g., polyhydroxybutyrate can be implemented in *E. coli*. PHA is a family of linear polyesters of hydroxy acids with a variety of thermoplastic properties and commercial uses. *Pseudomonas aeruginosa* genes encoding 3-ketoacyl coenzyme A reductases and poly-3-hydroxyalkanoate polymerase can be placed under regulation of a desired promoter, e.g., *glnAp2*, since acetyl-  
20 CoA levels can be indicative of precursor availability for PHA synthesis.

Without further elaboration, it is believed that the above description has adequately enabled the present invention. The following examples are, therefore to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are hereby incorporated by reference in their  
25 entirety.

### **Methods**

**Growth conditions.** All *E. coli* strains were grown in shake flasks containing the designated medium at 37°C in waterbath shakers (Model G76; New Brunswick Scientific, Edison, NJ). The cultures were grown in minimal media consisting of either M9 defined salts 34  
30 containing 0.5% (wt/vol) glucose or YE defined salts containing 1.5% (wt/vol) glucose. YE

defined salts consisted of (per liter) 14 g  $K_2HPO_4$ , 16 g  $KH_2PO_4$ , 5 g  $(NH_4)_2SO_4$ , 1 g  $MgSO_4$ , and 1 mg thiamine. Cell turbidity was monitored spectrophotometrically at 550 nm.

**Metabolite measurements.** Acetate, pyruvate, and other organic acids were measured using HPLC (Constametric 3500 Solvent Delivery System and Spectromonitor 3100 Variable Wavelength Detector; LDC Analytical, Riviera Beach, FL) over an organic acids column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA) maintained at 65°C. The mobile phase consisted of 0.01 N  $H_2SO_4$ , and its flow rate was kept at 0.6 ml min<sup>-1</sup>. Peaks coming off the column were detected at 210 nm. Glucose was measured using Sigma kit no. 315-100. To quantify lycopene, 1 ml of bacterial culture was extracted with acetone, centrifuged, and the supernatant absorbance was measured at 474 nm. Lycopene concentrations were calculated by comparing absorbances to a standard curve.

**SDS-PAGE and enzyme assays.** The protocol for SDS-PAGE is as described by Laemmli (1970) *Nature* 227:680-685. Measurement of  $\beta$ -galactosidase activity was carried out essentially as described by Miller (1992) *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

## **Results**

### **Usage of the *glnAp2* promoter in *E. coli* in a heterologous fusion to *lacZ*.**

Increasing levels of acetyl phosphate can be an indicator of excess glucose flux. The current invention features host cells, nucleic acids sequences, and methods of utilizing acetyl phosphate as a signal to regulate the expression of rate-controlling enzymes in a desired metabolic pathway, both to utilize fully the excess carbon flux and to redirect the flux away from the toxic product, acetate.

In order to examine the potential of *glnAp2* as a dynamic controller of product expression, a nucleic acid sequence was constructed containing a heterologous *lacZ* gene operably linked to the *glnAp2* promoter. The *glnAp2* promoter region containing the promoter and two ntrC-binding sites can be easily obtained by standard methods known in the art. The *glnAp2* promoter was PCR-amplified from *E. coli* genomic DNA using the forward primer 5'-CAGCTGCAAAGGTCATTGCACCAAC (containing an engineered *PvuII* site) and the reverse primer 5'-GGTACCAGTACGT-GTTCAGCGGACATAC (containing an engineered *KpnI* site). These two primers amplified a region between positions 93 and 343 in the published DNA sequence 16 (GenBank accession No. M10421).

The *glnAp2* PCR fragment was also cloned into the *EcoRI* site of pRS551, thus generating p2GFPuv, which contains *glnAp2* in front of a promoterless *lacZ* gene. The *glnAp2-lacZ* region was transferred to  $\lambda$ RS45 via homologous recombination (Simons *et al.* (1987) *Gene* 53:85-96), generating phage  $\lambda$ p2GFPuv. JCL1595 and JCL1596 were  
 5 constructed by integrating a *glnAp2-lacZ* fusion via infection (Silhavy *et al.* (1984) *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY) with  $\lambda$ p2GFPuv phage into the chromosomes of BW13711 (*lacX74*) and BW18302 (*lacX glnL2001*; Feng *et al. supra*), respectively. This strain contains the *glnL2001* allele,  
 10 which consists of an internal deletion between codons 23 and 182 of the *glnL* coding sequence and presumably results in a null mutation (Feng *et al. supra*).

The time course of the  $\beta$ -galactosidase ( $\beta$ -gal) activity was measured in wild-type and in the *glnL* mutant. The *glnAp2*- $\beta$ -gal activity increases in a time-dependent fashion similar to the excreted acetate concentration from the *glnL* host (JCL1596), whereas no induction of promoter activity was found for the isogenic wild-type control (JCL1595).

15 **Table 1.**  $\beta$ -galactosidase activity of *glnAp2-lacZ*

|   | $\beta$ -galactosidase activity<br>(nmol/min-mg protein) |          |
|---|--|----------|
|   | 6 hours  | 11 hours |
| <i>glnAp2-lacZ</i> in WT (JCL1595)          | <100   | ~100     |
| <i>glnAp2-lacZ</i> in <i>glnL</i> (JCL1596) | ~700   | ~1500    |
| <i>P<sub>lac</sub>-lacZ</i> in (VJS632)     | ~500   | ~550     |

Thus, in the absence of *glnL*, *glnAp2* is capable of responding to the excess carbon flux that is indicated by acetate excretion. As the cells approached the late-exponential phase, the biosynthetic requirement decreased and the cells began to exhibit an excess carbon flux, as demonstrated by the increased generation of acetate. At this point, at approximately 6  
 20 hours, unexpectedly *glnAp2*- $\beta$ -gal activity began to rise to (~700 nmol/min-mg protein, see Table 1) whereas *glnAp2*- $\beta$ -gal activity in the wild-type strain (JCL1595) was relatively low and remained constant throughout (~100 nmol/min-mg protein, Table 1). After more than 10 hours, *glnAp2*- $\beta$ -gal activity in the absence of *glnL* was a remarkable ~1500 nmol/min-mg protein (Table 1). The induction profile of *glnAp2* is also in stark contrast to that of the *lac*  
 25 promoter (*P<sub>lac</sub>*). Chromosomal *P<sub>lac</sub>* activity in strain VJS632 (*lac*<sup>+</sup>) rapidly increased after

induction with IPTG (isopropyl- $\beta$ -D-thio-galactopyranoside) and achieved a constant level of expression in the cell ( $\sim 550$  nmol/min-mg protein, see Table 1), which is independent of the growth phase.

## 5 Usage of the *glnAp2* promoter in *E. coli* in a heterologous fusion to *pps* and *aroG*

Expression of two different metabolic enzymes, phosphoenolpyruvate synthase (*pps*) and 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (*aroG*) were placed under the control of the *glnAp2* promoter. As controls, these same two proteins also were overexpressed from the *tac* promoter ( $P_{tac}$ ), which exhibits static control, under the same  
10 genetic background and environmental conditions. Standard methods of expressing *pps* leads to growth retardation (Patnaik *et al.* (1992) *J Bacteriol* 174:7527-7532).

Plasmid pAROG was constructed by cloning a PCR fragment containing *aroG* pRW5*tkl* into the *EcoRI*-*Bam*HI sites of pJF118EH. Plasmid pPS706 has been previously described in Patnaik *et al. supra*. Both plasmid express the respective genes under the  $P_{tac}$   
15 promoter. The PCR fragment containing the *glnAp2* promoter was cloned into the *EcoRV*-*EcoRI* sites of plasmids pAROG, and pPS706 to generate plasmids p2AROG3, and pPSG706, respectively containing the respective genes under the *glnAp2* promoter.

Host strain BW18302 (*lacX glnL2001*) was transformed with all four plasmids. The strains with the respective plasmids were grown in M9 salts-glucose media. Growth was  
20 compared after 5 hours.

**Table 2.** Growth of Overexpressing Strains

|                             | OD <sub>550</sub> after 5 hours growth |
|-----------------------------|--|
| No plasmid                  | $\sim 0.5$                             |
| $P_{tac}$ - <i>aroG</i>     | $\sim 0.5$                             |
| <i>glnAp2</i> - <i>aroG</i> | $\sim 0.5$                             |
| $P_{tac}$ - <i>pps</i>      | $\sim 0.12$                            |
| <i>glnAp2</i> - <i>pps</i>  | $\sim 0.4$                             |

As previously demonstrated, overexpression of *pps* using  $P_{tac}$ -*pps* caused marked growth retardation. However, the use of *glnAp2* unexpectedly resulted in close to normal  
25 growth (Table 2). After 15 hours, proteins were isolated from each strain and analyzed on a

10% SDS-PAGE gel. At least 500% more pps protein was expressed when the *pps* gene was controlled by the *glnAp2* promoter compared to the *P<sub>tac</sub>* promoter. In another surprising finding, AroG protein, whose conventional overexpression is not overtly detrimental, was also at least 300% more abundant in extracts from cells utilizing *glnAp2* promoter for  
5 expression compared to the *P<sub>tac</sub>* promoter.

### Production of Lycopene in *E. coli* by *idi* Overexpression

We reconstructed a recombinant lycopene pathway in *E. coli* by expressing the genes *dxs* (coding for 1-deoxy-D-xylulose 5-phosphate synthase) from *E. coli*, *gps* (coding for  
10 geranylgeranyl diphosphate (GGPP) synthase) from *Archaeoglobus fulgidus*, and *crtBI* (coding for phytoene synthase and desaturase, respectively) from *Erwinia uredovora*. These genes were inserted into pCL1920, a low-copy-number plasmid, to form pCW9, and simultaneously overexpressed.

We used the *glnAp2* promoter to control the expression of *idi* (isopentenyl  
15 diphosphate isomerase). Constructs containing the *idi* gene were derived from a promoterless vector, pJF118. The *glnAp2* promoter was inserted to form p2IDI. As a control, the *P<sub>tac</sub>* promoter was inserted to form pTacIDI. These plasmids were separately introduced into a *glnL* strain (BW18302) containing pCW9. The p2IDI-containing strain (*glnAp2-idi*) produced 100 mg L<sup>-1</sup> lycopene after 26 h in a defined medium containing glucose. The strain  
20 containing *P<sub>tac</sub>-idi* on the other hand, produced only a small amount of lycopene, (< 5 mg L<sup>-1</sup>) under identical conditions. Additionally, the p2IDI strain produced almost threefold less acetate than pTacIDI, which indicates that the carbon flux to acetate was being rechanneled to lycopene.

**Table 3.** Carbon yield of lycopene formation in batch cultures of *E. coli*.

|  | Lycopene Carbon yield on glucose<br>(mol C/mol C) |
|--|---|
| Host only (BW18302)  | 0.0000  |
| + pTacIDI ( <i>P<sub>tac</sub>-idi</i> )   | 0.0003  |
| + pTacIDI ( <i>P<sub>tac</sub>-idi</i> ) / pPS184 ( <i>P<sub>tac</sub>-pps</i> ) | 0.0012  |
| + p2IDI ( <i>glnAp2-idi</i> )  | 0.014   |
| + p2IDI ( <i>glnAp2-idi</i> ) / pPSG184 ( <i>glnAp2-pps</i> )                    | 0.022   |

### Use of *pps* to Enhance Lycopene Yield

*pps* was overexpressed from *glnAp2* from another compatible plasmid, pPSG18 while the remainder of the lycopene pathway (*dxs*, *gps*, *crtBI*) was expressed using pCL1920.

- 5 Coexpression of *pps* and *idi* with the lycopene pathway increased the final titer of lycopene by 50% and caused the productivity to increase by threefold, from 0.05 mg mL<sup>-1</sup> h<sup>-1</sup> to 0.16 mg mL<sup>-1</sup> h<sup>-1</sup> (Table 3) This is in contrast to the companion strain containing both pTacIDI and pPS184 (*P<sub>tac</sub>-idi* + *P<sub>tac</sub>-pps*), where no significant improvement in yield was observed and substantial growth inhibition occurred.

10

### Additional Host Cells for Lycopene Production

The *pykF::cat* and *pykA::kan* alleles were introduced into a wild-type strain, in order to generate two single mutants (JCL1610 (*pykF*) and JCL1612 (*pykA*)) and one double mutant strain (JCL1613 (*pykF pykA*)) (Ponce *et al.* (1995) *J Bacteriol* 177:5719-5722). The double

15 mutant strain was able to achieve a final lycopene titer of about 14 mg lycopene/g dried cells, while the single mutant strains each obtained lycopene titers of about 2.5 mg lycopene/g dried cells. The single *pyk* mutants produced lycopene at a level similar to the wild type strain, ~ 4 mg lycopene/g dried cells. Further, overexpression of Pck, phosphoenolpyruvate carboxykinase, increased the final lycopene titer by about 3-fold. Overexpression of Ppc,

20 phosphoenolpyruvate carboxylase, reduced lycopene production by about 30%.

### Other Embodiments

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and the scope of the present invention. Accordingly, other embodiments are within the scope of

25 the following claims. For example, all homologs of the mentioned polypeptides and genes are within the scope of this invention.



What is claimed:

1. A bacterial host cell comprising a nucleic acid sequence comprising a promoter and  
5 nucleic acid sequence encoding a heterologous polypeptide; the nucleic acid sequence being operably linked to the promoter which is controlled by a response regulator protein; the host cell being genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation.
2. The host cell of claim 1 wherein the bacterial cell is an *E. coli* cell.
- 10 3. The host cell of claim 1 wherein the promoter is controlled by a response regulator protein selected from the list consisting of ntrC, phoB, phoP, ompR, cheY, creB, and torR.
4. The host cell of claim 3 wherein the promoter is bound by ntrC.
5. The host cell of claim 4 wherein the promoter is *glnAp2*.
6. The host cell of claim 1 wherein the host cell is genetically modified by deletion or  
15 mutation of a gene encoding a histidine protein kinase.
7. The host cell of claim 6 wherein the histidine protein kinase is encoded by glnL.
8. The host cell of claim 1 wherein the heterologous polypeptide is a biosynthetic enzyme required for production of a metabolite.
9. A host cell comprising a first expression cassette comprising a promoter and a nucleic  
20 acid sequence encoding a first enzyme required for biosynthesis of a heterologous metabolite; the nucleic acid sequence being operably linked to the promoter which is regulated by acetyl phosphate in the absence of nitrogen starvation; and nucleic acid sequences expressing other enzymes required for biosynthesis of the metabolite.
10. The host cell of claim 9 wherein the metabolite is an isoprenoid.
- 25 11. The host cell of claim 10 wherein the isoprenoid is a carotenoid.
12. The host cell of claim 10 wherein the isoprenoid is lycopene,  $\beta$ -carotene, astaxanthin, or one of their precursors.

13. The host cell of claim 10 wherein the first enzyme is isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, or 1-deoxyxylulose 5-phosphate synthase.
14. The host cell of claim 9 wherein the first enzyme is phosphoenolpyruvate synthase.
15. The host cell of claim 9 wherein the host cell is a bacterial cell.
- 5 16. The host cell of claim 15 wherein the bacterial cell is an *E. coli* cell.
17. The host cell of claim 15 wherein the cell is lacking a functional histidine protein kinase gene.
18. The host cell of claim 15 wherein the promoter is controlled by *ntrC*, *phoB*, *ompR*, *cheY*, *creB*, *phoP*, or *torR*.
- 10 19. The host cell of claim 18 wherein the promoter is bound by *ntrC*.
20. The host cell of claim 19 wherein the promoter is *glnAp2*.
21. The host cell of claim 10 wherein the host cell further contains a second expression cassette comprising a nucleic acid sequence encoding a phosphoenolpyruvate synthase operably linked to a promoter which is regulated by acetyl phosphate concentration.
- 15 22. A method of producing heterologous isoprenoids in a host cell comprising overexpressing a heterologous phosphoenolpyruvate synthase; and expressing biosynthetic enzymes required for synthesis of the heterologous isoprenoid.
23. A method of producing a lycopene in a host cell comprising expressing a heterologous 1-deoxy-D-xylulose 5-phosphate synthase, a heterologous geranylgeranyl diphosphate
- 20 synthase, a heterologous phytoene synthase, and a heterologous phytoene desaturase.
24. A kit comprising a nucleic acid sequence containing a promoter controlled by a response regulator protein such that the promoter is regulated by acetyl phosphate in a defined host cell; and the defined host cell which is genetically modified by deletion or mutation of a histidine protein kinase gene.
- 25 25. A nucleic acid sequence comprising a promoter and a sequence encoding a biosynthetic enzyme required for the production of a first metabolite, the sequence being operably linked to the promoter which is regulated by a second metabolite whose concentration is indicative of availability of a precursor for the biosynthesis of the first metabolite.

26. The nucleic acid sequence of claim 25 wherein the second metabolite is a waste product produced from a precursor for the biosynthesis of the first metabolite.
27. The nucleic acid sequence of claim 25 wherein the first metabolite is an isoprenoid.
28. The nucleic acid sequence of claim 27 wherein the isoprenoid is a carotenoid.
- 5 29. The nucleic acid sequence of claim 28 wherein the isoprenoid is lycopene,  $\beta$ -carotene, astaxanthin, or one of their precursors.
30. The nucleic acid sequence of claim 25 wherein the second metabolite is acetyl phosphate, cAMP, fructose 1-phosphate, or fructose 6-phosphate.
31. The nucleic acid sequence of claim 30 wherein the second metabolite is acetyl phosphate.
- 10 32. The nucleic acid sequence of claim 31 wherein the promoter is controlled by ntrC, phoB, ompR, cheY, creB, phoP, or torR.
33. The nucleic acid sequence of claim 32 wherein the promoter is bound by ntrC.
34. The nucleic acid sequence of claim 33 wherein the promoter is *glnAp2*.
- 15 35. The nucleic acid sequence of claim 27 wherein the biosynthetic enzyme is isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, 1-deoxyxylulose 5-phosphate synthase, or phosphoenolpyruvate synthase.

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
1 February 2001 (01.02.2001)

PCT

(10) International Publication Number  
**WO 01/07567 A1**

- (51) International Patent Classification<sup>7</sup>: **C12N 1/21**,  
15/52, C12P 23/00, 5/02, C12Q 1/68
- (21) International Application Number: PCT/US00/20519
- (22) International Filing Date: 27 July 2000 (27.07.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/145,801 27 July 1999 (27.07.1999) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 60/145,801 (CIP)  
Filed on 27 July 1999 (27.07.1999)
- (71) Applicant (for all designated States except US): **FOOD INDUSTRY RESEARCH AND DEVELOPMENT INSTITUTE** [—/—]; P.O. Box 246, Hsinchu 300 (TW).
- (72) Inventor; and  
(75) Inventor/Applicant (for US only): **LIAO, James, C.** [—/US]; 10573 Wellworth Avenue, Los Angeles, CA 90024 (US).
- (74) Agent: **TSAO, Y., Rocky**; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110 (US).
- (81) Designated States (national): CN, JP, US.
- (84) Designated States (regional): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
- Published:**  
— With international search report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/07567 A1

(54) Title: **ENGINEERING OF METABOLIC CONTROL**

(57) Abstract: The invention features a method of producing heterologous molecules in cells under the regulatory control of a metabolite and metabolic flux. The method can enhance the synthesis of heterologous polypeptides and metabolites.

**COMBINED DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled ENGINEERING OF METABOLIC CONTROL, which was filed on January 25, 2002 as Application Serial No. 10/048,186.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim the benefit under Title 35, United States Code, §119(e)(1) of any United States provisional application(s) listed below:

| U.S. Serial No. | Filing Date   | Status  |
|-----------------|---------------|---------|
| 60/145,801      | July 27, 1999 | Expired |

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

| Country | Application No. | Filing Date   | Priority Claimed |
|---------|-----------------|---------------|------------------|
| WIPO    | PCT/US00/20519  | July 27, 2000 | [X] Yes    [] No |

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Y. Rocky Tsao, Reg. No. 34,053  
 Eric L. Pahl, Reg. No. 32,590  
 Harold H. Fox, Reg. No. 41,498

Frank R. Occhiuti, Reg. No. 35,306  
 John F. Hayden, Reg. No. 37,640  
 Jeffrey D. Hsi, Reg. No. 40,024

Address all telephone calls to Y. ROCKY TSAO at telephone number (617) 542-5070.

Address all correspondence to Y. ROCKY TSAO at:

~~ESH & RICHARDSON P.C.~~  
~~225 Franklin Street~~  
~~Boston, MA 02110-2804~~

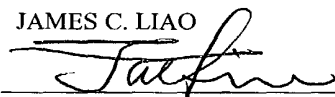
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section

**Combined Declaration and Power of Attorney**

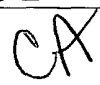
Page 2 of 2 Pages

1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Full Name of Inventor: JAMES C. LIAO

Inventor's Signature: Date: 5/15/2002

Residence Address:

10573 Wellworth Avenue  
Los Angeles, CA 90024 

Citizenship:

United States of America

Post Office Address:

Same As Above

20434989 doc